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Developing Molecular Tools for Diagnosis of Human Ehrlichiosis

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INTRODUCTION

Ehrlichia chaffeensis, *E. ewingii*, *E. muris* and *E. canis* are known to cause febrile illnesses in humans and dogs. These pathogens are transmitted by Ixodid ticks, including *Amblyomma americanum, Ixodes scapularis* and *Rhipicephalus sanguineus*. Infection is established following ehrlichia invasion of the human or dog white blood cells followed by general symptoms including headache, fever, fatigue, and muscle aches. *Ehrlichia chaffeensis* infections of humans have a fatality rate up to 5%, or higher in immunocompromised individuals.

Ehrlichiosis became a reportable disease in 1999. Since then, the number of reported cases has grown exponentially, classifying ehrlichiosis as an emerging infectious disease. Ehrlichiosis caused by *Ehrlichia ewingii* became a reportable disease in 2008.

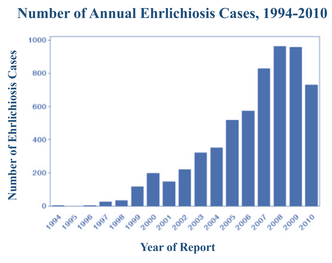

Figure 1: Increase in number of reported ehrlichiosis cases, Source: Centers for Disease Control.

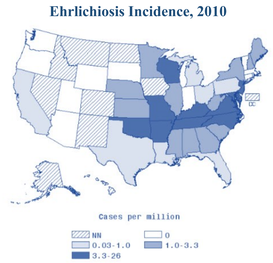

Figure 2: Distribution of reported ehrlichiosis cases by state, Source: Centers for Disease Control.

Current Detection Methods

The first week of ehrlichial infections is a critical time span for proper diagnosis of infection and for starting antibiotic treatment. Typically, hospitals use blood culturing methods for detection of bacterial infections but this approach is not suitable for obligate intracellular bacteria such as *Ehrlichia*. Ehrlichioses are diagnosed by PCR detection from a sample of whole blood during the first week of infection. Analysis of a blood smear for morulae can suggest species specific detection; *E chaffeensis* infects monocytes, whereas *E. ewingii* generally infects granulocytes. After the first week of infection, anti-ehrlichia antibodies may become detectable. The indirect immunofluorescent assay (IFA) for *E. chaffeensis* IgG is the primary detection method in hospitals for ehrlichiosis. The downside to this test is the need for paired serum samples taken over multiple week periods. A "positive" test for ehrlichiosis requires a 4 fold titer increase over 2-4 weeks of paired serum samples. After infection, antibody levels may not decrease for months after treatment. *Ehrlichia ewingii* displays antigenic cross-reactivity with *E. chaffeensis,* making the IFA test nonspecific and hard to interpret when both agents are present in the same area. There is no specific serological test for *E. ewingii* because this ehrlichia has not yet been cultivated.

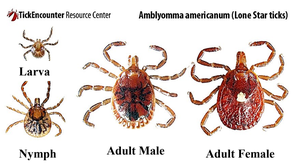

Figure 3: Life stages of *Amblyomma americanum*, a vector of ehrlichiosis. Source: Tick Encounter Resource Center, University of Rhode Island.

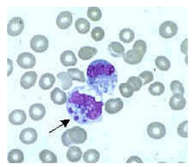

Figure 4: *E. chaffeensis* morulae in the cytoplasm of a human monocyte. Morulae is indicated by an arrow. Source: Centers for Disease Control.

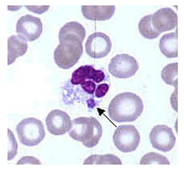

Figure 5: *E. ewingii* morulae in the cytoplasm of a neutrophil. Morulae is indicated by an arrow. Source: Centers for Disease Control.

Project Goals

Our project will develop approaches and tools to characterize the *E. ewingii* surface protein gene homologous to the major 120 kDa (*gp*120) diagnostic protein antigen gene of *E. chaffeensis* using a targeted RNA bait hybridization protocol.

APPROACHES AND RESULTS

Primers were designed using the OligoPerfect™ Designer from Thermo Fisher Scientific Inc. for long-range tile amplification of 900-3,000 base pair fragments of *E. chaffeensis*

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PCR Amplification of 18 fragments gave only partial coverage of the target fragment.

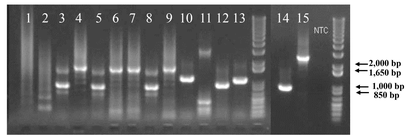

Fig. 6. Agarose gel of PCR amplified fragments with *E. chaffeensis* primers. Each lane depicts the amplification product yielded with each primer pair tested. From left to right, Fragment 1 through Fragment 13, ladder, Fragment 14, 15, NTC: Negative Control. Numbers on the side of the gel correspond to the sizes of the 1Kb Plus DNA ladder.

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More primers were designed to achieve full coverage of the target fragment..

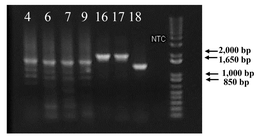

Fig. 7. Agarose gel of Hot Start PCR amplified fragments with *E. chaffeensis* primers. From left to right, Fragment 4, 6, 7, 9, 16, 17, 18, NTC: Negative Control. Numbers on the side of the gel correspond to the sizes of the 1Kb Plus DNA ladder.

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PCR conditions were optimized using Hot Start PCR to reduce non-specific amplification.

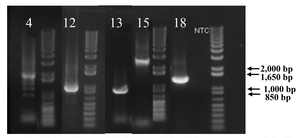

Fig. 8. Agarose gel of Hot Start PCR amplified fragments with *E. chaffeensis* primers. From left to right, Fragment 4, 12, 13, 15, 18, NTC: Negative Control. Numbers on the side of the gel correspond to the sizes of the 1Kb Plus DNA ladder.

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Five primer pairs were selected for full coverage of the gp120 gene.

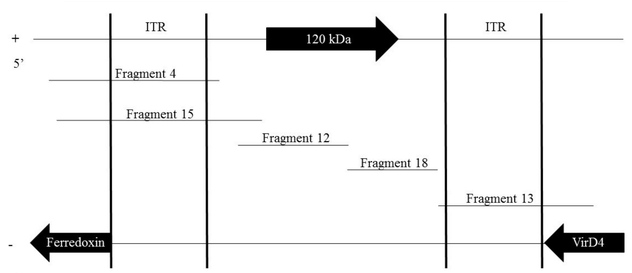

Fig. 9. Schematic diagram of *E. chaffeensis* gp120 gene and flanking genes and mapping of overlapping fragments for making capture baits. The map is not to scale.

5 Amplified fragments were cloned into a linearized pMiniT™ vector and transformed into *E. coli*.

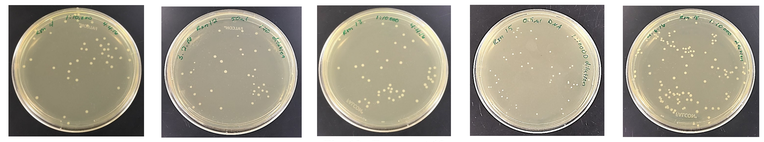

Fig. 10. Fragment 4 clones at 1:10000 dilution.

Fig. 11. Fragment 12 clones at 1:10000 dilution.

Fig. 12. Fragment 13 clones at 1:10000 dilution.

Fig. 13. Reaction 15 clones at 1:10000 dilution.

Fig. 14. Reaction 18 clones at 1:10000 dilution.

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Individual clones were screened by PCR using plasmid flanking primers and fragment sizes were examined by agarose gel electrophoresis

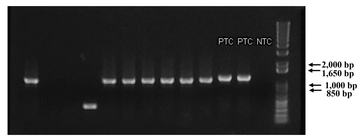

Fig. 15. Agarose gel of 10 individual clone colonies using *E. chaffeensis* Fragment 12 primers. NTC: Negative Control. PTC: Positive Control. Numbers on the side of the gel correspond to the sizes of the 1Kb Plus DNA ladder.

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Target fragments were re-amplified from selected clones and their identity was confirmed by Sanger sequencing.

FUTURE WORK

Selected pairs will be used to produce biotinylated RNA baits.

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Biotinylated *E.chaffeensis* baits will be used in a capture hybridization assay using a streptavidin bead pull down method to separate the *E. ewingii* gene targets from unwanted background DNA.

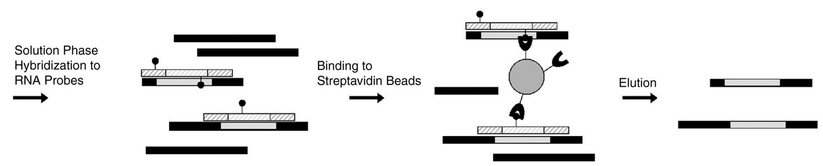

Fig. 16. Schematic diagram of a capture hybridization assay using streptavidin beads and biotinylated RNA baits. Black and grey bars represent gene targets, and fully black bars represent unwanted background DNA. Source: Summerer, D. 2009.

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Captured DNA will be purified and sequenced for characterization of the *E. ewingii* gp120 gene.

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ACKNOWLEDGMENTS

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